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REPAIR OF DNA MUTAGENIC DAMAGE

The present invention relates to the use of equol, dehydroequol and isoflav-3-ene and isoflavan compounds in promoting repair of DNA mutagenic damage.

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Metallothioneins (MT) are proteins synthesised or over expressed in response to DNA damaging agents e.g. UVR (Hansen et al 1997). In most of the studies in animals and tissue cultures, high doses of radiation were used to induce MT, and therefore, it is difficult to extrapolate these results to low level or repeated exposures to UVR in humans (Cai et al 10 1999). Induced synthesis of MT is considered as one of the mechanisms involved in the adaptive response to low dose UVR exposure, and increased levels of MT appear to be associated with protection from UVR, possibly mediated through scavenging of ROS in the skin (Hanada, et al 1992). As well, MT is implicated in protecting against the immunosuppressive effects of UVR on cell-mediated responses as demonstrated in MT=I 15 and II knockout mice (Reeve, et al 2000). UVR induces immunohistochemically detectable MT in keratinocytes and dermal fibroblasts concurrently with the photoconduction of p53, which suggests that these protein systems are protective and complimentary in function. MT is detectable in dermal fibroblasts from 2 hours post-UV (Anstey, et al 1996).

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Equol, dehydroequol, isoflav-3-ene and isoflavan compounds and methods for producing the same are described in copending International Patent Application PCT/AU03/00427 and WO 98/08503 which are incorporated herein by reference.

25 UV exposed skin causes damage in DNA which may give rise to carcinogenesis. The most common tumour in humans is the basal cell carcinoma (BCC) followed by squamous cell carcinoma (SCC), and more rarely malignant melanoma.

It has now been found by the applicant that compounds of the present invention, when 30 applied to the skin, result in elevation of metallothioneins production in the skin, particularly the basal layer of irradiated skin.

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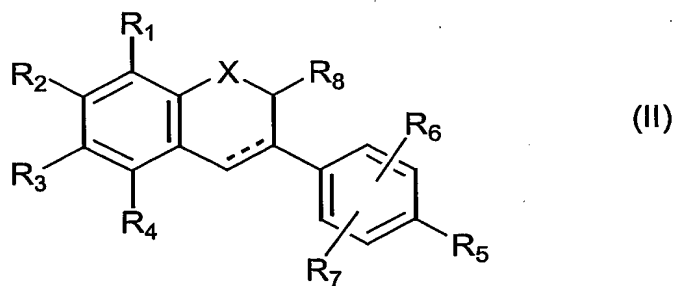
As mentioned above, metallothioneins affect and promote repair of DNA mutagenic damage of skin subject to UV exposure, and/or enhancing defence against UV-induced DNA mutagenic damage in skin.

- 5 In accordance with the present invention there is provided use of equol, dehydroequol, isoflav-3-ene or isoflavan structures for protecting skin from DNA mutagenic damage associated with UV exposure.

10 In another aspect there is provided use of equol, dehydroequol, isoflav-3-ene or isoflavan structures for the over expression of metallothioneins in the skin, particularly the basal layer of skin.

15 In accordance with another aspect of this invention there is provided a method for protecting skin from UV induced DNA mutagenic damage which comprises applying to skin a composition containing one or more of equol, dehydroequol, isoflav-3-ene, or isoflavan compounds in admixture with a dermally acceptable carrier.

Isoflav-3-ene and isoflavan compounds may be represented by the general formula (II)



20 in which

25 R_1 , R_2 , R_3 and R_4 are independently hydrogen, hydroxy, OR_9 , $OC(O)R_{10}$, $OS(O)R_{10}$, CHO , $C(O)R_{10}$, $COOH$, CO_2R_{10} , $CONR_{11}R_{12}$, alkyl, haloalkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, alkylaryl, alkoxyaryl, thio, alkylthio, amino, alkylamino, dialkylamino, nitro or halo, or

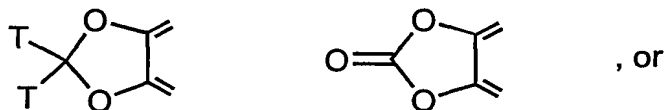
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R_3 and R_4 are as previously defined, and R_1 and R_2 taken together with the carbon atoms to which they are attached form a five-membered ring selected from



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R_1 and R_4 are as previously defined, and R_2 and R_3 taken together with the carbon atoms to which they are attached form a five-membered ring selected from



10

R_1 and R_2 are as previously defined, and R_3 and R_4 taken together with the carbon atoms to which they are attached form a five-membered ring selected from



15

and

wherein

R_5 , R_6 and R_7 are independently hydrogen, hydroxy, OR_9 , $OC(O)R_{10}$, $OS(O)R_{10}$, CHO , $C(O)R_{10}$, $COOH$, CO_2R_{10} , $CONR_{11}R_{12}$, alkyl, haloalkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, thio, alkylthio, amino, alkylamino, dialkylamino, nitro or halo,

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R_8 is hydrogen, hydroxy, alkyl, aryl, amino, thio, $NR_{11}R_{12}$, $CONR_{11}R_{12}$, $C(O)R_{13}$ where R_{13} is hydrogen, alkyl, aryl, arylalkyl or an amino acid, or CO_2R_{14} where R_{14} is hydrogen, alkyl, haloalkyl, aryl or arylalkyl,

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- R₉ is alkyl, haloalkyl, aryl, arylalkyl, C(O)R₁₃ where R₁₃ is as previously defined, or Si(R₁₅)₃ where each R₁₅ is independently hydrogen, alkyl or aryl,
- R₁₀ is hydrogen, alkyl, haloalkyl, amino, aryl, arylalkyl, an amino acid, alkylamino or dialkylamino,
- 5 R₁₁ is hydrogen, alkyl, arylalkyl, alkenyl, aryl, an amino acid, C(O)R₁₃ where R₁₃ is as previously defined, or CO₂R₁₄ where R₁₄ is as previously defined,
- R₁₂ is hydrogen, alkyl or aryl, or
- R₁₁ and R₁₂ taken together with the nitrogen to which they are attached comprise pyrrolidiny1 or piperidiny1,
- 10 the drawing "___" represents either a single bond or a double bond, preferably a double bond,
- T is independently hydrogen, alkyl or aryl, and
- X is O, NR₁₂ or S, preferably O,
- including pharmaceutically acceptable salts and derivatives thereof.
- 15
- Equol corresponds to the formula (II) when R₁, R₂, R₃, R₄, R₆, R₇ and R₈ are hydrogen, R₅ is hydroxy, X is O, and "___" is a single bond. Dehydroequol corresponds to formula (II) when R₁, R₂, R₃, R₄, R₆, R₇ and R₈ are hydrogen, R₅ is hydroxy, X is O and "___" is a double bond.
- 20
- Dermally acceptable carriers and lotions are well known in the art, and are described for example in *Remington's Pharmaceutical Sciences*, Gennaro A. 18th Ed., Mack Publishing Co., Easton, PA, 1990, pp. 1492-1517. Any dermatologically acceptable carrier can be used in the compositions of the invention. As used herein, "dermatologically acceptable
- 25 carrier" refers to vehicles, diluents, carriers, which can include adjuvants, additives, or excipients, known for use in dermatological compositions. The compositions of the invention include, but are not limited to, creams, ointments, solutions, sticks, wipes, cleansers and/or gels. The compounds of the present invention may be simply mixed, admixed or blended with suitable carriers to give compositions suitable for application to
- 30 the skin. Dermally acceptable carriers may include one or more sunscreen agents. Sunscreens include those materials commonly used to block ultraviolet light. Illustrative

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compounds include the derivatives of cinnamate, PABA, and salicylate. For example, octyl methoxycinnamate and 2-hydroxy-4-methoxy benzophenone (also known as oxybenzone) can be used. Octyl methoxycinnamate and 2-hydroxy-4-methoxy benzophenone are commercially available under the trademarks, Parsol MCX and Benzophenone-3, respectively. The exact amount of sunscreen employed can vary depending upon the degree of protection desired from the sun's UV irradiation.

In a preferred embodiment one or more compounds of the formula (II) are formulated into cosmetic preparations. Examples of cosmetic formulations include creams, gels, powders, pastes, cakes and the like. Typically such cosmetics may be referred to as "make-up", and/or foundation (typically used to provide a smooth, even appearance to skin and as a base for coloured cosmetics).

Compounds of the formula (II) may be used in the compositions in an amount from 0.001% to 100%, preferably from 0.1% to 20%, most preferably from 0.1% to 10% w/w. For example, compositions may comprise 1 μ m to 500 mmol equol or other compounds of the formula (II), such as 20 μ m to 400 μ m. The remainder of the composition will comprise one or more dermatologically acceptable carriers and excipients as are well known in the art. One or more compounds may be utilised in the compositions, with equol and dehydroequol being particularly preferred. Compositions may be administered topically to the skin before, during and/or after sun exposure. Typically, doses of between about 1 to 500 mg per day, with doses between 2 to 100 mg per day being preferred.

In accordance with another aspects of this invention there is provided a method for the treatment, or amelioration or preventing the formation of skin cancer, such as basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma, which comprises applying to the skin of a subject a composition containing one or more of equol, dehydroequol, or an isoflav-3-ene or isoflavan compounds of the general formula (II).

In another aspect of this invention there is provided a method for increasing metallothionein production in the skin, such as the basal layer of skin, which comprises

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applying to skin one or more of equol, dehydroequol, isoflav-3-ene or isoflavan compound in association with a dermally acceptable carrier.

The applicant has further found that the compounds according to this invention promote
5 DNA repair. The promotion of DNA repair may be by one or more of increasing the rate of repair of cyclobutane pyrimidine dimers (CPDs), promoting DNA repair by decreasing P53 expression, and/or by promoting the formation of metallothionein (MT).

The formation of CPD is considered to be an important lethal and mutagenic consequence
10 of UVR exposure (Mitchell et al, 1989; Liardet et al, 2000). Animal models have demonstrated an inverse relationship between epidermal CPD repair and skin carcinogenesis (Young et al, 1996). The P53 protein (TP53) is expressed after DNA damage by UV irradiation. P53 is a transcription factor which blocks cellular progression from G1 to S phase, thus preventing replication of damaged DNA (Campbell et al, 1993).
15 The P53 protein may act as a tumour promoting agent (Murphey et al, 2001).

This invention will be described with reference to the following, non-limiting examples.

Example 1

20 The effect of equol on the induction of CPD was examined in the skin of hairless mice (a standard model for human dermatological investigations) exposed to solar simulated ultraviolet radiation (SSUV). At various time points after SSUV, dorsal skin was excised, fixed for 6hr in a standard fixing medium (HistoChoice™, Amersco Inc, Solon, Ohio, USA), processed and paraffin-embedded. Pyrimidine dimers were detected
25 immunohistochemically using citric acid antigen retrieval and the H3 anti-pyrimidine dimer antibody. The number of dimer-positive cells was counted manually in 30 fields per mouse, at 40x magnification.

When equol lotion (containing 20 µM equol) was applied daily for 7 days prior to and
30 following irradiation with 1 x 3MED of SSUV, the effect of equol was to reduce the initial

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induction of dimers, and to enhance the rate of their repair, as evidenced by a reduced number of dimers at 24 hr (Table 1).

Table 1: Induction of epidermal CPD-positive cells following UV irradiation

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Time of collection	Treatment	CPD +ve cells/linear cm
	Normal skin	0
1hr post-SSUV	Vehicle + SSUV	300 ± 18
	equol + SSUV	238 ± 22
24hr post-SSUV	Vehicle + SSUV	340 ± 55
	equol + SSUV	167 ± 17

Application of equol immediately after SSUV exposure (and continuing for 5d) resulted in significantly reduced dimers at 1 day post-irradiation (a significant reduction of 23%), and at 2d (a significant reduction of 42% -data not shown).

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When equol lotion (20µM) was applied for both 7 days prior and 5 days after SSUV exposure, the reduction in CPD numbers was evident immediately and at 1, 24 and 48 hours after ($p < 0.05$; 54%, 50% and 26% reduction in the number of CPD respectively) compared with the control group (vehicle alone).

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Example 2

Equol was applied to the skin of five human volunteers immediately after, and at 4 hours and 6 hours post-UV irradiation. A control lotion was also used containing no equol. Twenty-four hours after UV irradiation, skin biopsies were taken and MT production was measured using immunohistochemistry.

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Table 2 shows the counts of cells in the basal epidermis and superficial dermis that demonstrated positive staining for MT. Approximately half of the cells in the basal epidermis constitutively expressed MT at baseline, whereas almost none of the cells in the

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more superficial layers of the epidermis expressed MT. At 24 hrs after exposure to 2.5 MED SSUV, there were apparent differences in the expression of MT in the basal layers of the epidermis between sections treated with equol and those treated with DMSO in base lotion (vehicle). In all 5 participants, the expression of MT was higher in the skin treated with equol, with the magnitude of the difference ranging from +4% to +21%.

Table 2: Proportion of cells staining positively for MT in the epidermis of five human volunteers, by treatment group

Subject	treatment	Total epidermis			Upper epidermis			Basal epidermis		
		neg	pos	%	neg	pos	%	neg	pos	%
NO1DWH	Baseline	303	201	40	99	0	0	204	201	50
	10 mins	255	179	41	72	0	0	183	179	49
	DMSO	282	185	40	70	0	0	212	185	47
	equol	303	382	56	185	2	1	118	380	76
NO3PPA	Baseline	227	109	32	97	0	0	130	109	46
	10 mins	231	237	51	77	4	5	154	233	60
	DMSO	317	236	43	96	4	4	221	232	51
	equol	270	271	50	82	0	0	188	271	59
NO6MED	Baseline	420	413	50	169	0	0	251	413	62
	10 mins	437	565	56	168	1	1	269	564	68
	DMSO	440	442	50	130	6	4	310	436	58
	equol	315	539	63	76	8	10	239	531	69
N13PDO	Baseline	267	217	45	112	0	0	155	217	58
	10 mins	468	703	60	270	10	4	198	693	78
	DMSO	465	405	47	144	0	0	321	405	56
	equol	323	527	62	169	5	3	154	522	77
N14GBO	Baseline	270	127	32	113	0	0	157	127	45
	10 mins	381	242	39	247	0	0	134	242	64
	DMSO	276	217	44	111	4	3	165	213	56
	equol	225	234	51	68	1	1	157	233	60

10 Note: "Baseline" refers to the skin sections from the punch biopsy taken prior to exposure to 2.5 MED SSUV.

"10 mins" refers to the skin sections from the punch biopsy taken 10 mins after exposure to 2.5 MED SSUV. The skin was not treated with either DMSO in base lotion (vehicle) or equol at 200 μ M.

15 "DMSO" refers to the skin sections from the punch biopsy taken 24 hrs after exposure to 2.5 MED SSUV. The skin was from the grid treated with DMSO in base lotion (vehicle).

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"Equol" refers to the skin sections from the punch biopsy taken 24 hrs after exposure to 2.5 MED SSUV. The skin was from the grid treated with equol at 200 μ M.

The increase of MT immunoreactivity in basal and suprabasal keratinocytes of recently
5 UV-exposed individuals was highest in skin that had been treated with equol.

Example 3

The skin biopsies from the five human volunteers from Example 2 were tested for cyclobutane pyrimidine dimer formation using immunohistochemistry.

10

Table 3 presents the counts and percentages of cells staining positively with an antibody directed against CPD. These data demonstrate that, as expected, there were essentially no CPD-positive cells in the epidermis prior to irradiation with 2.5 MED. However, skin sections taken from all of the participants 10 mins after UV exposure showed high levels
15 of DNA damage, with the proportion of positively-staining cells ranging from 36% (participants N01DWH and N03PPA) to 87% (participant N14GBO).

Skin sections taken 24 hrs after UV exposure showed substantially lower levels of CPD damage in all subjects. For 4 out of 5 participants, the skin sections treated with equol
20 lotion had proportionally less CPD-positive cells than the skin sections treated with DMSO in base lotion (vehicle).

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Table 3: Proportion of cells staining positively for CPDs in the epidermis of five human volunteers, by treatment group

Subject	treatment	Total epidermis			Upper epidermis			Basal epidermis		
		neg	pos	%	neg	pos	%	neg	pos	%
NO1DWH	Baseline	345	0	0	134	0	0	211	0	0
	10 mins	162	107	40	64	51	44	98	56	36
	DMSO	231	105	31	81	47	37	150	58	28
	equol	164	39	19	70	23	25	94	16	15
NO3PPA	Baseline	309	0	0	104	0	0	205	0	0
	10 mins	204	191	48	56	106	65	148	85	36
	DMSO	179	25	12	55	20	27	124	5	4
	equol	349	18	5	70	17	20	279	1	0
NO6MED	Baseline	309	0	0	90	0	0	219	0	0
	10 mins	136	364	73	19	198	91	117	166	59
	DMSO	339	65	16	112	59	35	227	6	3
	equol	279	92	25	98	71	42	181	21	10
N13PDO	Baseline	205	0	0	60	0	0	145	0	0
	10 mins	69	195	74	20	94	82	49	101	67
	DMSO	105	68	39	78	51	40	27	17	39
	equol	213	94	31	79	50	39	134	44	25
N14GBO	Baseline	255	0	0	98	0	0	157	0	0
	10 mins	34	389	92	0	157	100	34	232	87
	DMSO	240	131	35	93	69	43	147	62	30
	equol	188	85	31	63	44	41	125	41	25

Note: "Baseline" refers to the skin sections from the punch biopsy taken prior to exposure to 2.5 MED SSUV. "10 mins" refers to the skin sections from the punch biopsy taken 10 mins after exposure to 2.5 MED SSUV. The skin was not treated with either DMSO in base lotion (vehicle) or equol at 200 μ M.

"DMSO" refers to the skin sections from the punch biopsy taken 24 hrs after exposure to 2.5 MED SSUV. The skin was from the grid treated with DMSO in base lotion (vehicle).

"Equol" refers to the skin sections from the punch biopsy taken 24 hrs after exposure to 2.5 MED SSUV. The skin was from the grid treated with lotion containing equol at 200 μ M.

When data from all participants were pooled, it can be seen that skin sections treated with equol had moderately lower levels of CPD damage at 24 hours.

Example 4

The skin biopsies from the five human volunteers from Example 2 were tested for P53 staining following UV irradiation. Results are shown in Table 4.

5 Table 4: Proportion of cells staining positively for p53 in the epidermis of five human volunteers, by treatment group

Subject	treatment	Total epidermis			Upper epidermis			Basal epidermis		
		neg	pos	%	neg	pos	%	neg	pos	%
NO1DWH	Baseline	261	0	0	115	0	0	146	0	0
	10 mins	343	1	0	154	1	1	189	0	0
	DMSO	187	12	6	79	4	5	108	8	7
	equol	270	94	26	109	48	31	161	46	22
NO3PPA	Baseline	274	2	1	112	1	1	162	1	1
	10 mins	316	2	1	114	2	2	202	0	0
	DMSO	223	55	20	81	31	28	142	24	14
	equol	337	87	21	165	72	30	172	15	8
NO6MED	Baseline	412	1	0	134	0	0	278	1	0
	10 mins	402	3	1	153	1	1	249	2	1
	DMSO	462	133	22	165	77	32	297	56	16
	equol	500	50	9	250	19	7	250	31	11
N13PDO	Baseline	325	0	0	141	0	0	184	0	0
	10 mins	304	0	0	140	0	0	164	0	0
	DMSO	222	45	17	109	8	7	113	37	25
	equol	287	13	4	147	4	3	140	9	6
N14GBO	Baseline	321	0	0	149	0	0	172	0	0
	10 mins	292	4	1	185	2	1	107	2	2
	DMSO	217	190	47	106	106	50	111	84	43
	equol	227	76	25	109	35	24	118	41	26

Note: "Baseline" refers to the skin sections from the punch biopsy taken prior to exposure to 2.5 MED SSUV.

10 "10 mins" refers to the skin sections from the punch biopsy taken 10 mins after exposure to 2.5 MED SSUV. The skin was not treated with either DMSO in base lotion (vehicle) or equol at 200 μ M.

"DMSO" refers to the skin sections from the punch biopsy taken 24 hrs after exposure to 2.5 MED SSUV. The skin was from the grid treated with DMSO in base lotion (vehicle).

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"Equol" refers to the skin sections from the punch biopsy taken 24 hrs after exposure to 2.5 MED SSUV. The skin was from the grid treated with equol at 200 μ M.

As expected, there were essentially no cells in the epidermis expressing p53 prior to irradiation with 2.5 MED for any of the participants. Similarly, skin sections taken from participants 10 mins after UV exposure showed negligible levels of p53 expression, in accordance with the literature.

Skin sections taken 24 hrs after UV exposure showed substantially higher levels of p53 expression in all subjects. The percentage of p53 expression in upper and/or basal epidermis was reduced in four out of five equol treated subjects. For example, in subjects N13PDO and N14GBO the percentage of p53 staining was reduced significantly (generally more than 50%) compared with vehicle controls.

15 Conclusions

The biomarkers assessed in these experiments were selected based on their biological associations with skin cancer (which is directly associated with UV-induced DNA mutagenic damage).

UV-induced oxidative damage is now recognised as a potentially important causal factor in skin cancer. MTs are molecules with anti-oxidant properties that are specifically induced in response to UV exposure. This study found consistent evidence that human skin treated with equol, and it is believed other compounds of the formula (II), induce more MT than skin treated with base lotion.

25

CPDs are the earliest indicator of molecular damage following exposure to UV radiation, and if not repaired, lead to fixed mutations in the DNA of skin cells. Thus one mechanism of action of a post-exposure treatment would be to increase the rate of repair of these lesions. The experiments conducted here suggest that CPD repair may be enhanced by topical equol compositions, and other compositions containing one or more compounds of the formula (II).

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P53 is clearly an important regulatory gene that is commonly mutated in epidermal skin cancers. Moreover, in normal skin cells, p53 is up-regulated following UV exposure to prevent mitosis until DNA damage is repaired. Equol modulated the expression of p53 in
5 this study causing a reduction in the number of cells in the upper or basal epidermis expressing p53 for four of five subjects.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be
10 understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as an acknowledgment or any form of suggestion that that prior art forms part of the common
15 general knowledge in Australia.

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